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Lack of correlation between DNA methylation and hepatocarcinogenesis in rats and hamsters treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

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Previous studies have demonstrated that the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induced liver tumors in F344 rats but not in Syrian golden hamsters. The aim of this study was to determine whether there was a correlation between the persistence of O^6-methylguanine (O^6-mGua) adducts and the rate of recovery of O^6-methylguanine-DNA methyltransferase (O^6-mGuaT) after depletion in the liver and susceptibility to NNK in F344 rat and Syrian golden hamster injected s.c. with NNK (80 mg/kg). The levels of both 7-methylguanine and O^6-mGua reached a maximum 24 h after NNK treatment. O^6-mGua in NNK-treated rat liver was undetectable after 48 h. In the rat, the depletion of O^6-mGuaT activity occurred within 4 h following NNK treatment. A subsequent rapid recovery of enzyme activity was observed 36 h after NNK exposure. In contrast, high levels of O^6-mGua persisted in hamster liver DNA and no O^6-mGuaT activity was detected up to 336 h after NNK injection. Thus, the persistence of O^6-mGua in hamster liver is most likely related to a lack of recovery of the O^6-mGuaT. These results suggested that factors other than O^6-mGua may be determining NNK-induced hepatocarcinogenesis in rats. An aldehyde generated by α-hydroxylation of NNK, 4-oxo-4-(3-pyridyl)butanal, inhibited O^6-mGuaT activity in rat hepatocytes, suggesting that this aldehyde contributes to the carcinogenicity of NNK by inhibiting this repair enzyme.

Introduction

The O^6-methylguanine (O^6-mGua*) DNA adduct repair process may play an important protective role in carcinogenesis that is initiated by methylating *N*-nitrosamines. In most mammalian tissues, this lesion is repaired by a suicide enzyme, the O^6-methylguanine-DNA methyltransferase (O^6-mGuaT) having the following characteristics: (i) the methyl group of an O^6-mGua residue in alkylated DNA is enzymatically transferred to a thiol group of the transferase molecule, leaving an intact guanine moiety; (ii) the transfer of the methyl group is rapid and results in the inactivation of the enzyme; (iii) each enzyme molecule stoichiometrically removes one methyl group from DNA. The constitutive levels of O^6-mGuaT determine the initial repair capacity (1). Following total depletion of the enzyme, the rate of repair of O^6-mGua will depend on the rate of synthesis of new enzyme molecules (2–5). Different constitutive levels of O^6-mGuaT exist in various types of cells, organs and species. In mammalian species, the highest level of O^6-mGuaT is present in hepatocytes (6).

Epidemiological studies in Japan (7) and the USA (8) have provided evidence that cancer of the liver is positively associated with cigarette smoking. The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is the most potent carcinogenic nitrosamine found in tobacco smoke and smokeless tobacco products (9). The activation of NNK is initiated by α-hydroxylation of the methylene (10) resulting in methyldiazohydroxide. This methylating species reacts with DNA to yield a variety of products including 7-methylguanine (7-mGua), O^6-mGua and O^4-methylthymidine (11,12). In rats, these adducts are present only in target tissues such as lung, liver and nasal mucosa (11,13,14).

Previous carcinogenicity studies in rats and hamsters have demonstrated that NNK frequently induces liver tumors in the rat but only rarely in the hamster (11,15–20). For instance, Hall *et al.* (21) demonstrated a good correlation between the persistence of O^6-mGua and hepatocarcinogenesis induced by a single dose of NDMA in these two species. Thus, we questioned whether susceptibility to NNK-induced hepatocarcinogenesis is linked to the persistence of O^6-mGua and the rate of recovery of O^6-mGuaT in these species. Little is known about the differences in the formation of O^6-mGua and the activity of O^6-mGuaT between NNK-treated F344 rats and Syrian golden hamsters. In fact, the rate of formation and removal of O^6-mGua in the liver of NNK-treated hamster has not been investigated. For this reason, we compared the level of persistent O^6-mGua adducts and the rate of recovery of O^6-mGuaT following NNK treatment in the F344 rat, which is NNK-sensitive, and in the Syrian golden hamster, which is NNK-resistant.

Materials and methods

Chemicals and reagents

NNK was synthesized (purity, 98%) as previously described (22). 4-Oxo-4-(3-pyridyl)butanal (OPB) was prepared as described by Abbaspour *et al.* (23) and stored at −20°C under a nitrogen atmosphere. The chemical structure and the purity of OPB was confirmed by NMR and TLC. OPB was dissolved in DMSO and freshly prepared before each experiment. NNK was dissolved in 0.9% NaCl (40 mg/ml). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO).

Animals

Male F344 rats and male Syrian golden hamsters (10 weeks old) were purchased from Charles River, Inc. (St Constant, Québec, Canada), fed a Rodent Laboratory Chow 5001 (Purina, St Romuald, Québec, Canada) and given tap water *ad libitum*. After a single s.c. injection of NNK (80 mg/kg body wt) in the thigh, the animals were anesthetized with ether and the livers were excised at various intervals. Liver tissues were immediately frozen and stored at −80°C until processed for enzyme preparation and DNA isolation.

Isolation and culture of hepatocytes

The rats were anesthetized with ether. Hepatocytes were isolated by a two-step collagenase perfusion as described previously (24,25). The cells were cultured under an atmosphere of 5% CO_2 and 95% air in α-minimum essential medium containing penicillin (50 U/ml) and streptomycin (50 μg/ml). Viability of hepatocytes was measured by trypan blue exclusion before and after treatment with tested compounds. Viability of cells was 90 ± 3%. Freshly isolated hepatocytes (2×10^6) were allowed to attach for 2 h in 10 ml α-minimum

*Abbreviations: O^6-mGua, O^6-methylguanine; O^6-mGuaT, O^6-methylguanine-DNA methyltransferase; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 7-mGua, 7-methylguanine; OPB, 4-oxo-4-(3-pyridyl)butanal.

essential medium plus 10% fetal bovine serum and washed once with α-minimum essential medium to remove dead cells before exposure to OPB or formaldehyde. The OPB and formaldehyde solutions were prepared in DMSO; the final concentration of DMSO in cultured medium was 0.5% (v/v). To measure the inhibition of O^6-mGuaT activity by OPB, hepatocytes were incubated in increasing concentrations of OPB or formaldehyde for 4 h, harvested and washed with PBS with 1 mM EDTA. Cells (10×10^6) were resuspended in 1 ml of cell extract buffer (70 mM HEPES, 0.1 mM EDTA, 5% glycerol and 1 mM dithiothreitol, pH 7.8) and frozen in liquid nitrogen until assayed for O^6-mGuaT.

Tissue and cell extracts

Liver tissue samples were homogenized in a Ten Broeck cell disrupter (Fisher Scientific, Pittsburgh, PA) at 4°C in 4 vol of cell extract buffer (26). Tissue homogenate and cell extracts were prepared by sonication at 4°C to complete cell disruption, followed by centrifugation at 12 000 *g* for 2 min to remove cellular debris.

DNA and protein assay

The DNA content of the cell extract was measured fluorimetrically using the fluorescence enhancer Hoechst 33258. Duplicate 15 μl aliquots of cell extract were mixed with 2 ml of DNA assay buffer (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). One milliliter of 1.5 μM Hoechst dye 33258 in DNA assay buffer was added to each tube. The fluorescence was measured with excitation at 360 nm and emission at 450 nm. A standard curve was established using 0.25–10 μg/ml of thymus DNA dissolved in DNA assay buffer. Protein content in the cell extract was assayed according to Lowry *et al.* (27).

O^6-mGuaT assay

O^6-mGuaT activity in tissue and cell extracts was measured by the amount of ^{3}H-methyl group removed from O^6-[^{3}H]methylguanine-DNA alkylated with *N*-[^{3}H]methyl-*N*-nitrosourea (6,26,28). The reaction mixture was incubated for 60 min at 37°C and stopped with 54 μl 50% trichloroacetic acid and incubated at 4°C for 30 min. The precipitate was collected by centrifugation (12 000 *g*, 2 min) and washed with 300 μl 80% ethanol. The pellet was hydrolyzed with 150 μl 0.1 N HCl at 80°C for 60 min and then neutralized to pH 7 with 375 μl 0.04 M Tris base. Hydrolyzed purines present in the supernatant were separated by HPLC on ODS 12.5 cm 5 μM partisil column (Whatman Inc., Clifton, NJ) and [^{3}H-methyl]O^6-mGua and [^{3}H-methyl]7-mGua peaks were quantified by liquid scintillation. One unit of alkyltransferase activity was defined as the removal of 1 fmol of O^6-mGua/μg cellular DNA. This expression adjusts for changes in DNA content of the cell occurring during the cell cycle and DNA synthesis.

DNA methylation assay

DNA was isolated from rat liver by chloroform/isoamyl alcohol extraction as described by Daoud and Irving (29). Assay of 7-mGua, O^6-mGua and guanine in DNA was performed by HPLC with UV and fluorescence detection as described previously (30). Levels of 7-mGua and O^6-mGua were measured by fluorimetry with excitation at 280 nm and emission at 360 nm. Guanine was measured by UV absorbance at 254 nm. Quantitations were based on standards run on the same day.

Results

Persistence of methylated guanine

The levels of O^6-mGua and 7-mGua in rat and hamster liver DNA were determined from 4 to 72 h following a single s.c. injection of NNK (80 mg/kg). As summarized in Figures 1 and 2, the initial levels of 7-mGua and O^6-mGua in both species were similar ($P < 0.2$). The maximum levels of 7-mGua and O^6-mGua in rat liver were reached 24 h after administration of NNK. The repair of O^6-mGua with a $t_{1/2}$ equal to 12 h was observed in rats. In contrast, O^6-mGua in the hamster liver was repaired rather slowly and only 14% of these adducts had been repaired 72 h after treatment. The 7-mGua persisted longer in hamster than in rat and was still present 72 h after the NNK injection in the hamster.

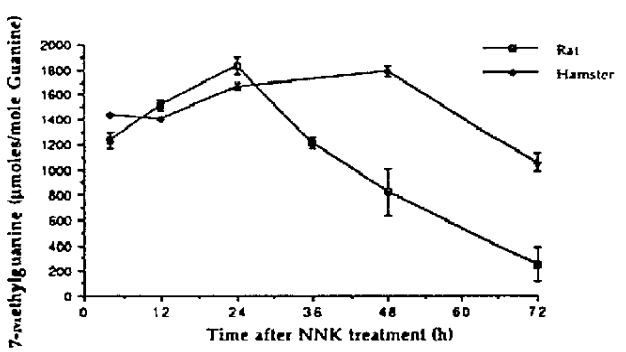

Fig. 1. Levels of 7-mGua in rat and hamster liver DNA after s.c. injection of 80 mg/kg of NNK. Each point is the mean of three determinations from three rats; bars, SE.

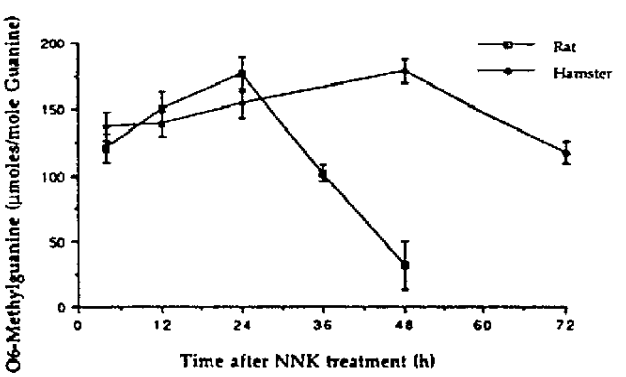

Fig. 2. Levels of O^6-mGua in rat and hamster liver DNA after s.c. injection of 80 mg/kg of NNK. Each point is the mean of three determinations from three rats; bars, SE.

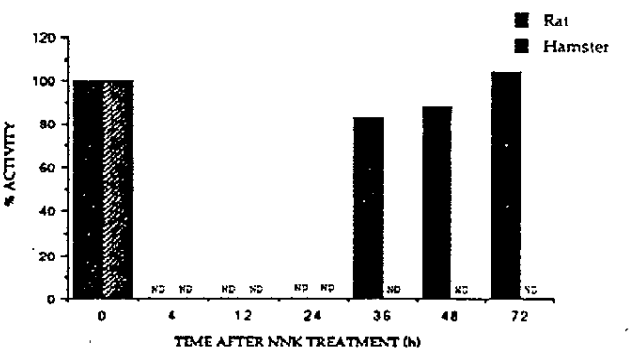

Fig. 3. Recovery of liver O^6-mGuaT activity following s.c. injection of NNK (80 mg/kg) to F344 rats or Syrian golden hamsters. One hundred per cent activity corresponds to 9.1 fmol O^6-Gua removed/μg DNA for the rat or 5.3 fmol O^6-mGua removed/μg DNA for the hamster. The limit of detection is 0.1 fmol/μg DNA.

Table I. O^6-mGuaT activity in rat hepatocytes treated with OPB

Treatment	Concentration (mM)	O^6-mGuaT activity (fmol O^6-mGua removed/μg DNA)
None (0.05% DMSO)		7.1 ± 0.6[a]
OPB	0.1	5.2 ± 0.8[b]
	0.5	2.4 ± 0.2[b]
	1.0	1.0 ± 0.7[b]
Formaldehyde	1.0	8.4 ± 0.2[a]

Inactivation of O^6-mGuaT was linear between 0.1 and 1.0 mM OPB ($r = 0.937$).
[a]Mean of three determinations ± SE obtained from each of three rats.
[b]Enzyme activity was statistically different from that of control cells incubated with 0.5% DMSO. $P < 0.01$ (Student's *t*-test).

Recovery of O^6-mGuaT in liver

O^6-mGuaT activity in liver extracts prepared from untreated rats and hamsters was 9.1 ± 0.7 and 5.3 ± 0.1 (mean ± SE, n = 3) fmol O^6-mGua removed/μg DNA respectively. The recovery of O^6-mGuaT activity in rats and hamsters following NNK treatment were qualitatively and quantitatively different. As shown in Figure 3, NNK-induced depletion of O^6-mGuaT in both species occurred within 4 h of NNK treatment. In rat liver the recovery of O^6-mGuaT activity started between 24 and 36 h after NNK injection and reached control levels 72 h after NNK treatment. In hamster, no O^6-mGuaT activity was detectable in liver extract even 336 h after NNK treatment (data not shown).

Effects of OPB and formaldehyde on O^6-mGuaT

Hepatocytes were isolated from rat liver and assayed for activity of O^6-mGuaT after exposure to formaldehyde and OPB. The inactivation of O^6-mGuaT activity following cells cultured with OPB at non-cytotoxic concentrations is shown in Table I. The inhibitory effect of OPB on O^6-mGuaT was correlated to the OBP concentration between 0.1 and 1.0 mM (r = 0.937) and reached maximal inhibition of 86% at 1.0 mM OPB. The specificity of this reaction is suggested by the fact that 1.0 mM formaldehyde did not inhibit O^6-mGuaT activity in hepatocytes *in vitro*.

Discussion

Good correlations have been found between the persistence of O^6-mGua in the DNA of animal tissues after exposure to an alkylating agent and susceptibility of that tissue to tumor development (1,21). Thus, comparing two species with different susceptibilities to NNK hepatic carcinogenesis will help to define the role of the formation and removal of O^6-mGua in tumor induction. Previous investigators have observed the presence of persistent O^6-mGua in target tissues of NNK-treated rats and have suggested that O^6-mGua formed after the activation of NNK was associated with NNK carcinogenesis (12,13). However, in this study we found that the persistence of O^6-mGua does not correlate with the hepatocarcinogenicity of NNK in rats and hamsters. This observation suggests that in rats factors other than O^6-mGua are crucial in NNK hepatocarcinogenesis and supports the previous conclusion that persistent O^6-mGua in replicating cells may be necessary but not sufficient for tumor development (1,31). Likewise, in the hamster, some factors protect the liver despite high levels of O^6-mGua and a low capacity for repair by O^6-mGuaT.

We found that rat liver repairs O^6-mGua more efficiently than hamster liver by using a protocol of NNK treatment that was identical to that used in the rat by Hecht *et al.* (11). The levels of 7-mGua were 10-fold greater than O^6-mGua and both O^6-mGua and 7-mGua in rat liver reached a maximum 24 h after NNK injection and then decreased. O^6-mGua in the rat was undetectable 72 h after NNK treatment. In hamster liver, maximum O^6-mGua levels were also reached 24 h after NNK, but, contrary to rat liver, they remained elevated and were removed much more slowly. In these two species, the time required for full recovery of O^6-mGuaT activity was markedly different. The activity of O^6-mGuaT from rat liver had recovered to control values 72 h after treatment with NNK. The observation of a slow removal of O^6-mGua in hamster liver is consistent with the observation that O^6-mGuaT activity remains low for 336 h after NNK treatment. Collectively, the persistence of O^6-mGua and the recovery of O^6-mGuaT activity measured in rat and hamster liver after a single dose of NNK s.c. injection are in line with results obtained with NDMA by Stumpf *et al.* (32) and Hall *et al.* (21). NNK (0.39 mmol/kg) and NDMA (0.34 mmol/kg) gave a similar profile of inhibition of rat liver O^6-mGuaT. Both carcinogens are activated via α-hydroxylation to methylating species resulting in the formation of O^6-mGua (10). In addition, NNK may also be activated to pyridyloxobutylation species (15). Further studies should determine whether this activation pathway is more important in rats than in hamsters.

In contrast to NDMA, NNK rarely induces liver tumors in hamsters, whereas it is a definite hepatic carcinogen in the rat. The species difference in the susceptibility to this hepatocarcinogen may lie in a difference in the susceptibility of oncogenes or suppressor genes to mutation by methylating and/or pyridyloxobutylating agents. For example, methylating agents activate *ras* and non-*ras* oncogenes in mouse and rat respectively (33–35). It is also possible that adducts such as O^4-methylthymidine are more persistent in rat than in hamster liver (23,36). Toxicity of NNK in both species has been reported (12,19). Differences in cell proliferation following NNK-induced necrosis could explain the species difference in the liver carcinogenesis.

OPB and formaldehyde are intermediates generated by α-hydroxylation of NNK. Previous studies in our laboratory have demonstrated that OPB acts directly to induce DNA damage during NNK metabolism (25,37). Our results show that formaldehyde has no detectable effect on O^6-mGuaT activity in rat hepatocytes. In contrast, OPB reduces the O^6-mGuaT activity in rat hepatocytes in a concentration-dependent fashion. This inhibition could result from the reaction of the carbonyl group with the sulfhydryl group of cysteine, which is located at the active site of O^6-mGuaT and functions as an acceptor of the methyl group from O^6-mGua (38,39). We suggest that OPB formed during the hepatic metabolism of NNK could contribute to the carcinogenicity of NNK by inhibiting the O^6-mGuaT, which in turn further reduces repair of O^6-mGua DNA adducts resulting from NNK activation.

In summary, this study suggests that the mechanism of NNK hepatocarcinogenesis is complex and that, unlike NDMA, differences in susceptibility between species cannot solely be ascribed to differences in repair of O^6-mGua–DNA adducts.

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